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14. ABSTRACT

Purpose: Our goal is to develop a light-activated technology with the potential to decrease vision loss and ocular complications in warfighters sustaining penetrating eye injuries. **Scope:** In year 1, the scope was to establish the treatment parameters for sealing amniotic membrane over penetrating corneal lacerations (ex vivo and in vivo) and to design a light delivery system that reduces retinal radiant exposure to below established safe limits. **Major findings:** We identified the treatment parameters (amnion patch size, dye concentration, dye staining time and the irradiance and fluence of green light) that strongly bonded Rose Bengal-stained amnion patches over corneal wounds in <u>ex vivo</u> rabbit eyes. Determined that 100 J/cm^2 of 532 nm laser light (6.5 min irradiation) forms a seal between amnion and cornea <u>in vivo</u> that resists opening at intraocular pressures up to 350 mm Hg. Designed and constructed optical system for direct light-activated bonding of penetrating injuries.

15. SUBJECT TERMS
cornea, sclera, eyelid skin, penetrating wound, laser, photochemistry

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INTRODUCTION

The overall goal of this research is to develop a light-activated technology with the potential to decrease vision loss and ocular complications in warfighters sustaining penetrating eye injuries. Fragments and debris propelled at high velocity by improvised explosive devices (IEDs) have increased the incidence of penetrating eye injuries in the current conflicts compared to earlier wars. Rapid closure of penetrating eye wounds with formation of a water tight seal is critical to preventing infection and stabilizing the eye for further surgery, thus improving vision outcomes. Suturing the cornea, sclera and eyelid skin requires specialized training to precisely place hair-fine sutures and requires long surgery time. Cyanoacrylate glues can complicate further surgery by sticking to sutures and possibly causing additional damage when removed. Our sutureless, glueless method (1-4) is rapid and uses currently FDA-allowed devices (clinical laser, light-activated dye, amniotic membrane) and thus may move rapidly to the deployment environment. The scope of the research includes evaluating two light-activated approaches to closing penetrating injuries in the cornea and sclera of rabbit eyes. In one method, amniotic membrane is stained with the dye, placed over the wound and treated with green light; in the other, the dye is applied to the wound walls and activated by green light to directly close the wound. The scope also includes developing a light-activated method for rapid closure of eyelid lacerations using hairless mouse skin as a model. Finally, the scope includes designing, constructing and evaluating a green laser light delivery system that meets ANSI standards for retina and iris safety. Major tasks for Year 1 were to establish the treatment parameters for sealing amniotic membrane over corneal lacerations (first ex vivo, then in vivo), to evaluate healing after wound sealing and to design a light delivery system that reduces radiant exposure at the retina to below the established damage threshold levels.

BODY

This research project is a collaboration between military medical investigators who treat battlefield eye wounds, a laboratory team who developed the light-activated treatment and investigators who have extensive experience in laser eye safety. This Grant Agreement is a joint proposal with Col Anthony J. Johnson, MD at Brooke Army Medical Center (BAMC) who is PI on Grant Agreement # WB1WH-09-2-0069. The Statement of Work includes tasks to be carried at both the Massachusetts General Hospital (MGH) and the BAMC. Dr. Kochevar and Dr. Johnson have discussed this project frequently by phone and during Dr. Johnson's visit to Dr. Kochevar's lab in Boston in April, he provided ophthalmologic expertise during an early in vivo study.

Task 1. Evaluate photoactivated bonding for sealing amniotic membrane over corneal lacerations

1.a. Obtain IACUC and ACURO approvals Approvals were obtained on July 31, 2009 (IACUC) and September 4, 2009 (ACURO) for studies at MGH and on March 6, 2009 (IACUC) and April 16, 2010 (ACURO) for studies at BAMC.

1.b. Establish parameters for strong bonding of amniotic membrane to cornea
Our approach was to first determine in isolated rabbit eyes the influence of several treatment parameters (dye concentration, dye staining time, amnion patch size, irradiance and fluence of green laser light) on the strength of the seal between amnion and cornea. We then carried out in vivo studies using treatment parameters selected on the basis of the ex vivo studies and measured the bonding strength immediately after treatment (non-survival surgery). The following methods were used for both ex vivo and in vivo studies.

Preparation of amniotic membrane -- Human placentas from scheduled Caesarian section deliveries were obtained with the approval of the Institutional Review Board of the Partners Healthcare System. After washing, the amnion was peeled away from the chorion. The epithelial cells were removed by treating with trypsin (0.25% solution, 90 min, 37°C incubator) and lightly rubbing with a tissue culture cell scraper. The amnion was placed on nitrocellulose paper and cut into ~ 5 cm x 5 cm segments. These segments were stored at -80°C in a 1:1 solution of glycerol and Dulbecco's Modified Eagle's Medium (DMEM) with 1% penicillin-streptomycin. Immediately prior to use, membrane segments were defrosted and rinsed in phosphate buffered saline (PBS) for 45 min to remove all glycerol. Circular patches (10 to 16 mm diameter) were cut from the membrane using a metal punch.

Rose Bengal (RB) preparation and amnion staining -- Rose Bengal (95%) was obtained from Sigma-Aldrich (St. Louis MO) and prepared as a 0.1% weight/volume solution in 0.1 M PBS. Sufficient RB solution (~200 µl) was placed on the stromal surface of an amnion patch (while attached to the nitrocellulose paper) for the appropriate length of time. Excess RB was removed and the amnion washed briefly with PBS.

Laser and light delivery optics -- A clinical laser, IRIDEX OcuLight OR KTP green laser (IRIDEX Corp., Mountain View CA), that emits cw 532 nm was used for all studies except for one study that required a higher irradiance (0.5 W/cm^2). For that experiment, a cw Nd/YAG laser (Aura i; Laserscope, San Jose CA) was used. For both lasers, the beam was transmitted with a 600 µm optical fiber to a microscope objective (20X or 40X). The uniformity of the beam for irradiations was measured by translating a 1-mm pinhole over a 2.5 cm diameter image of the beam on a large area NOVA power meter (Ophir Optronics Ltd., North Andover MA) and shown to vary less than 11%. The microscope objective was held at a fixed distance from the cornea to produce the appropriate beam diameter.

Irradiation procedure -- An amnion patch (10 to 16 mm diameter) was placed with the stromal-side, i.e., the RB-stained side, in contact with the cornea and smoothed out to remove wrinkles while being observed through a dissecting microscope (Figure 1). A 4-mm diameter pupil-blocking opaque white cardboard disc was placed over the center cornea. The laser spot size at the level of the cornea was adjusted to be slightly larger than the diameter of the amnion patch. The irradiance was 0.25 W/cm^2 except as noted. The eye was misted with water every 90 sec (at ~ 25 cm distance) during the irradiation to prevent drying.
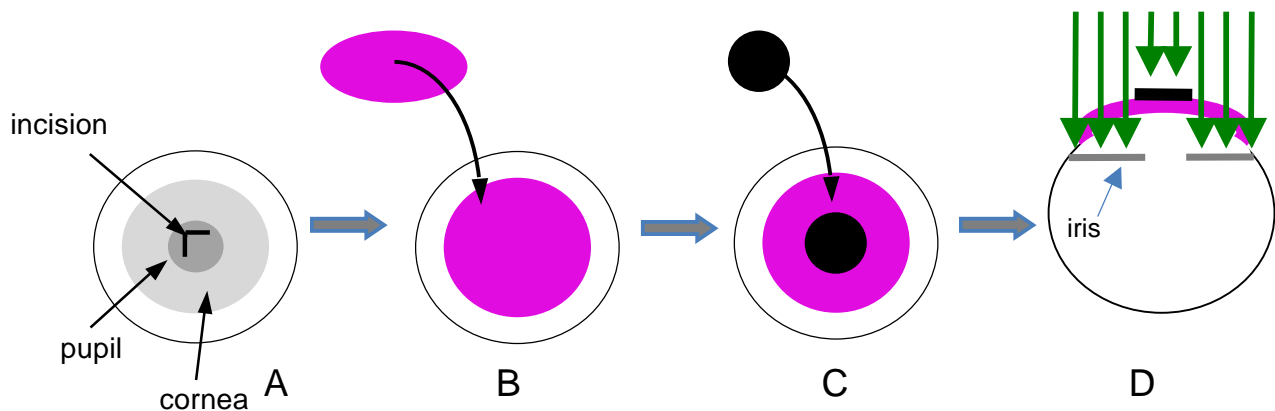

Figure 1. (A) An incision is made in the central cornea. (B) A Rose Bengal-stained amnion patch is placed over the cornea. (C) An opaque disc is placed over the pupil. (D) The eye is irradiated with green (532 nm) laser light (side view).

Measuring bond strength -- The strength of the bonding between the amnion and cornea after treatments was determined by measuring the intraocular pressure (IOP) that causes leakage through the incision (the IOP_L). A 22G needle was inserted into the clear cornea ~1 mm anterior of the corneal limbus and parallel to the iris. The needle was connected to both a mini-infuser (GENIE Plus Infusion/Withdrawal Pump, Kent Scientific, Torrington CT) and an ISOTEC pressure transducer (Harvard Apparatus, Holliston MA) via a T-couple. Saline was infused into the anterior chamber at 1.5 ml/min. The pressure attained immediately before fluid leaked from under the amnion (the leak intraocular pressure, IOP_L) was used as the measure of the bonding strength.

Statistical analysis –The student t-test for unpaired data was used for intergroup comparisons with significance set at $p < 0.05$.

1.b.i. <u>Ex vivo</u> bonding of amnion to rabbit cornea
The goal of these studies was to establish the treatment parameters to be used in subsequent <u>in vivo</u> studies of light-activated bonding of amnion patches to rabbit cornea. The influences of amnion patch size, dye concentration, dye staining time, irradiance and fluence were measured by systematically varying these parameters.

Preparation of <u>ex vivo</u> rabbit eyes -- Frozen albino rabbit eyes were purchased from Pel-Freeze Biologicals (Rogers AR) and allowed to reach room temperature in warmed PBS. To remove the epithelial layer, the corneal surface was placed in 80% ethanol for 10-15 seconds and the layer was removed with microforceps. The corneal surface was soaked in PBS for a few minutes to remove remaining ethanol, then dried. A V-shaped blade (90° angle, 2-mm arms), was used to make two incisions in a V-shape in the central cornea. The incision walls were approximately perpendicular to the cornea surface. A small amount of fluid was released from the anterior chamber to reduce the pressure and inhibit leakage during the irradiation. A 4 mm diameter opaque disc was placed on the central cornea unless noted otherwise (Figure 1).

Amnion patch size -- The effect of the size of the amnion patch on the bonding strength was assessed first. Circular amnion patches having diameters of 10, 13 and 16 mm, were stained with 0.1% RB, then bonded to rabbit corneas <u>ex vivo</u> using a fluence of 100 J/cm². The 10 mm diameter amnion covered the cornea except for a ~ 1 mm rim, the 13 mm amnion completely covered the cornea and the 16 mm diameter amnion extended over the sclera by ~1-1.5 mm. The IOP_L measured after irradiation did not differ significantly ($p > 0.05$) between amnion patches with the three different diameters; 10 mm = 139 ± 117 (n= 11), 13 mm = 144 ± 49 (n = 6) and 16 mm = 175 ± 83 (n = 5) mm Hg. <u>We selected the 13-mm diameter amnion patch for the following studies</u> because this size would cover injuries over a larger portion of the corneal surface than the 10 mm amnion patch and because light delivery was more uniform for the 13 than the 16 mm patch, which, because it followed the curvature of the globe, received less light nearer the perimeter.

Concentration of Rose Bengal solution and staining time -- The amniotic membrane absorbs and concentrates RB because this dye strongly associates with collagen fibers in the stromal layer (5). Since higher RB concentrations absorb more light, the RB level is expected to influence the bonding strength. The RB concentration in the amnion was varied by treating the stromal surface of amnion patches with RB (0.05 and 0.1%, ~200 µl) for 5, 10, 15, 20 or 25 min. Absorption spectra for these membranes are shown in Figure 2. The absorption at 532 nm approximately doubled between 5 and 10 min of incubation with either RB concentration and increased further after 15 min incubation. The results at longer incubation times not shown

because the RB within the membrane absorbs more than 99% of the 532 nm light from reaching the amnion/cornea interface where bonding occurs. Thus, these concentrations could not be used for bonding.

The absorbance of amnion stained with 0.05% RB after 5 min was 0.50 at 532 nm, indicating that ~ 32% of the light reaches the bottom of the amnion where bonding occurs; for 0.10% RB the absorbance of 1 at 532 nm indicates that only 10% of the light reaches the bottom of the amnion. However, this is counterbalanced by the fact that at the higher RB concentration, more RB molecules are present at the bottom of the amnion patch to absorb the 532 nm light. To determine which of these two effects dominates for bonding, we used the same light fluence (100 J/cm^2) and irradiance (0.25 W/cm^2) and 5 min incubations in 0.10% or 0.05% RB. As shown in in Figure 2C, the IOP_L produced using 0.05% RB did not differ from that produced by 0.10% RB. Apparently, the two effects of RB concentration are approximately equally balanced. Thus, for practical purposes in the following studies, the amnion patches were stained with 0.1% RB for 5 min.
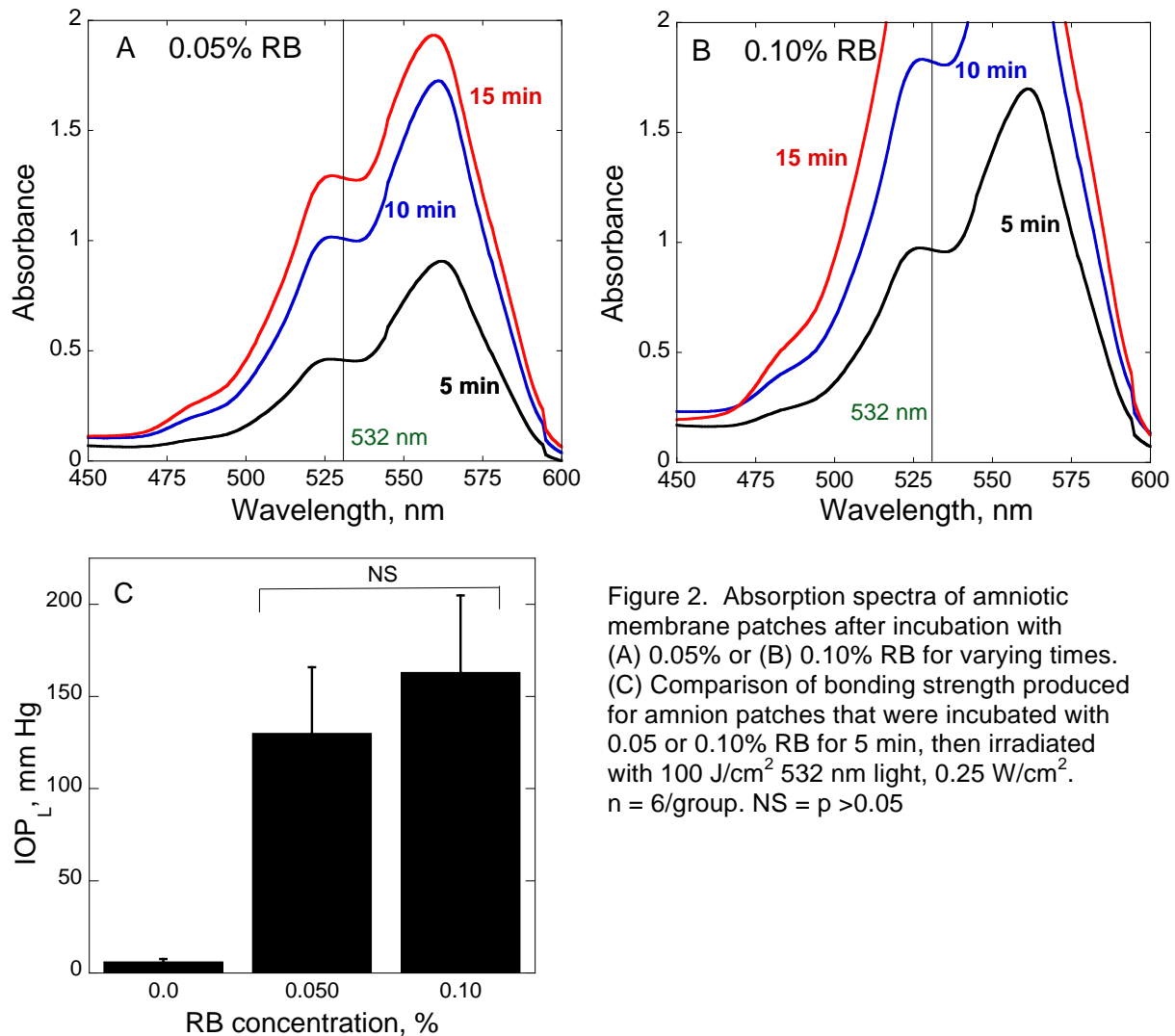

Figure 2. Absorption spectra of amniotic membrane patches after incubation with (A) 0.05% or (B) 0.10% RB for varying times. (C) Comparison of bonding strength produced for amnion patches that were incubated with 0.05 or 0.10% RB for 5 min, then irradiated with 100 J/cm^2 532 nm light, 0.25 W/cm^2. n = 6/group. NS = p >0.05

Irradiance of 532 nm light -- The light delivery parameters, irradiance (rate of light delivery to an area) and fluence (the total amount of light delivered per area) are related by:

Fluence (J/cm^2) = Irradiance (W/cm^2) x Time (sec)

Thus, using a higher irradiance allows the same fluence to be delivered in a shorter time, an advantage in a clinical setting. However, a higher irradiance also can increase the temperature when the energy is delivered more rapidly than it can be dispersed by convection or conductance in the tissue.

We measured the influence of irradiance on bond strength using 0.125, 0.250 and 0.500 W/cm^2 with amnion incubated 5 min with 0.1% RB and a fluence of 100 J/cm^2. The control was amnion without RB but irradiated at 0.125 W/cm^2. The temperature was measured during the irradiation with a Fluke 572 infrared thermometer. The results in Figure 3 indicate that increasing the irradiance by a factor of 4 did not change the bonding strength; no significant differences were found between the three experimental groups.
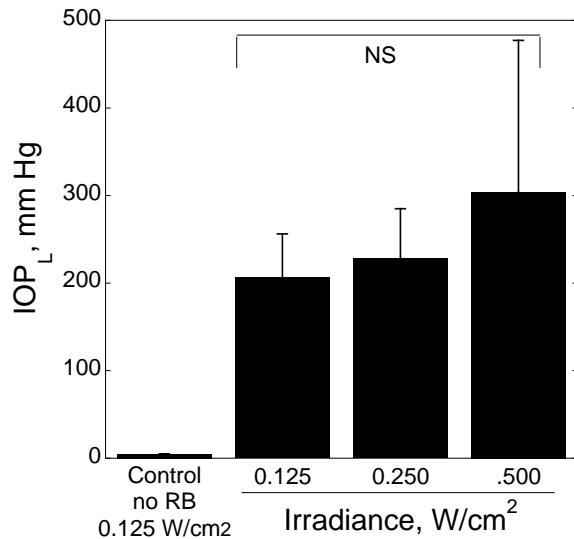

Figure 3. Relationship between irradiance and bonding strength. Treatment parameters were 0.5% RB, 100 J/cm^2. Control corneas were covered with amnion without RB and irradiated at 0.125 W/cm^2. n = 6. NS = p > 0.05 for all pairwise comparisons at the different irradiances.

The temperature did not increase from 18.3°C during the control irradiation. The maximum temperatures attained during the irradiation corneas treated with irradiances of 0.125, 0.250 and 0.500 W/cm^2 were 22.2, 27.8 and 36.7°C, respectively, indicating that a substantial increase is only observed at the highest irradiance. However, this temperature, 36.7°C, is much lower that is required for bonding using thermal laser welding (~63°C) (6) supporting a photochemical mechanism for light-initiated bonding using our treatment parameters rather than a thermal mechanism. This result is consistent with our previous measurements using RB-stained ex vivo skin in which 0.56 W/cm^2 increased the temperature to 40°C (3, 7). In order to minimize any potential thermal effect during bonding, we used 0.25 W/cm^2 in the following studies.

Fluence of 532 nm light -- Irradiating with increasingly higher fluences is expected to increase the bonding strength until all the potential crosslinking sites have reacted. Therefore, we next measured the relationship between fluence (50, 100 and 150 J/cm^2) and bonding strength using 0.1% RB and an irradiance of 0.25 W/cm^2. Control 1 was RB stained amnion without irradiation and Control 2 was amnion without RB but treated with 150 J/cm^2. As shown

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in Figure 4, the IOP_L values for Control 1 and Control 2 were 5 ± 3 and 10 ± 9 mm Hg, respectively, and were significantly lower than the IOP_L produced by the three experimental fluences. The bonding strength increased as the fluence increased. The IOP_L produced by 100 and 150 J/cm^2 differed significantly (p = 0.03), but there was no significant difference between the IOP_L measured after 50 and 100 J/cm^2. Even the IOP_L at 50 J/cm^2 (95 mm Hg) is substantially higher than normal human IOP (~20 mm Hg). Although these results suggest that fluences higher than 150 J/cm^2 might produce even stronger bonding, the time required to deliver the light becomes impractical. Delivering 150 J/cm^2 at 0.25 W/cm^2 requires 10 min, which is a long time in clinical situation. Fluences of 50 and 100 J/cm^2 are delivered in 3.33 and 6.67 min, respectively. <u>However, since the efficiency of bonding may differ between ex vivo and in vivo corneas, all three fluences were evaluated in vivo, as described below.</u>
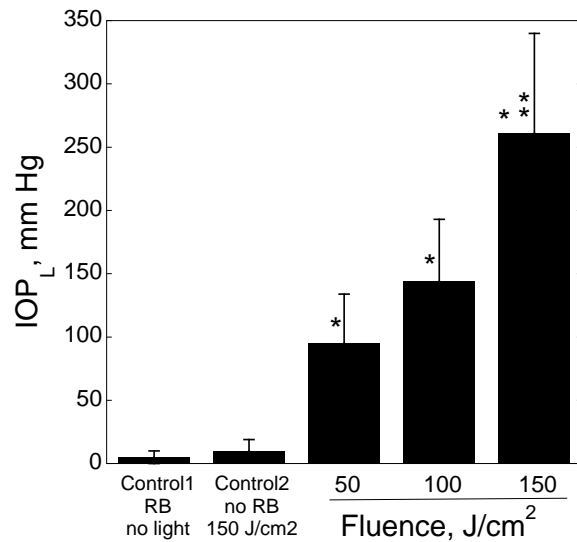

Figure 4. Relationship between fluence of 532 nm light and bonding strength measured by the intraocular pressure before leak (IOP_L) for amnion bonded to cornea ex vivo. RB = 0.1%, irradiance = 0.25 W/cm^2. n = 6 /group.
 * indicates p < 0.05 versus control 2. ** indicates p < 0.05 versus 100 J/cm^2 group.

Improving the efficiency of light-activated bonding -- Our previous studies indicated that PTB bonds two tissue surfaces by forming chemical links (photo crosslinks) between proteins (largely collagen) on the tissue surfaces. We may be able to increase the efficiency (thus decrease the irradiation time) by understanding mechanisms for the photo crosslinking reactions and possibly adding accelerators. We have begun experiments to identify steps in the reaction mechanism. First we examined whether the presence of oxygen influenced bonding strength since photoexcitation of RB leads to formation of reactive oxygen species that may be involved in the bonding mechanism (8, 9). We compared the IOP_L produced when the irradiation was carried out in air, nitrogen or oxygen atmospheres. The atmosphere was controlled by gas flow in a specially designed irradiation chamber. Eyes to be irradiated were placed in the chamber, which was then purged with the appropriate gas for 10 min before beginning the irradiation and during the irradiation.

The cornea was stained with 1.0% RB for 2 min and a 3.5 mm linear incision was made in the central cornea. A fluence of 100 J/cm^2 and irradiance of 0.25 W/cm^2 were used to treat all corneas (n = 5 /group). Control corneas were irradiated in an oxygen atmosphere but were not stained with RB. In air and oxygen atmospheres, the IOP_L values were 178 ± 12 and 208 ± 81 mm Hg, similar to the bond strength produced in the previous experiments (Figs. 3 and 4). Irradiation of corneas in a nitrogen atmosphere produced a substantially lower bonding strength than in air (Figure 5). The IOP_L after treatment in a nitrogen atmosphere was not significantly different from the IOP_L for the no RB control. These results indicate that oxygen is required in

the mechanism for photo-crosslink formation that produces strong bonding between amnion and cornea.
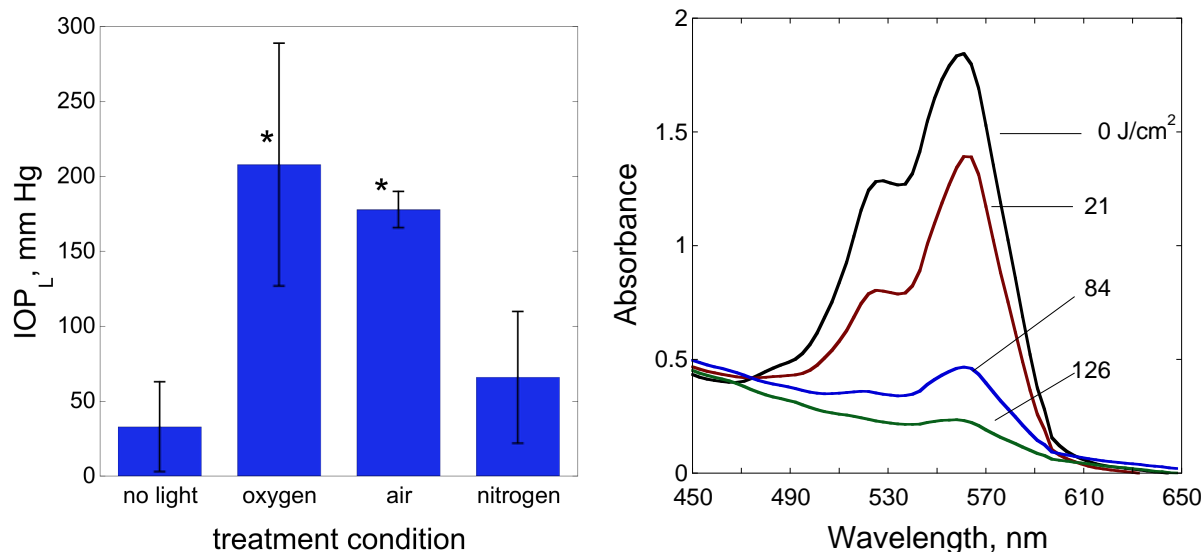

Figure 5. (A) Influence of atmosphere photoactivated bonding. After conditioning eyes with oxygen, air or nitrogen, RB-stained amnion patches on corneas were irradiated with 100 J/cm^2 532 nm light. N = 4/group. * indicates p <0.05 compared to no-RB control. (B) Photobleaching of RB during irradiation of RB-stained amnion patches.

We also began investigating the photodestruction (photobleaching) of RB during the irradiation because as RB is destroyed, the efficiency of photobonding is reduced. We examined the photobleaching by recording the absorption spectra of amnion stained with 0.1% RB and irradiated with fluences between 21 and 126 J/cm^2. As shown in Figure 5B, substantial photobleaching occurred; at 84 J/cm^2 the absorption decreased by over 70%. We will continue this investigation by carrying out the irradiation of RB-stained amnion while in place on the cornea and irradiate with 0, 50, 100 and 150 J/cm^2 and testing potential inhibitors of the photobleaching.

In summary, the results of these <u>ex vivo</u> studies identified the PTB treatment conditions (0.1% RB, 5 min stain, irradiance = 0.25 W/cm^2, fluences = 50 - 150 J/cm^2, 13 mm amnion patch, 4 mm opaque disc) that produce strong bonding of amnion to isolated rabbit eyes. These conditions will be used to evaluate bonding strength after PTB in vivo.

1.b.ii. <u>In vivo</u> bonding of amnion to rabbit cornea
The goal of this study was to establish the 532 nm fluence that produces strong bonding of amnion patches to rabbit cornea <u>in vivo</u>. This fluence will be then be used in subsequent healing studies after bonding to be conducted at BAMC. Our approach is to use the treatment parameters identified in the systematic studies using <u>ex vivo</u> rabbit eyes (described above) with three fluences of green (532 nm) light. In essence, this approach allows us to translate the results of the <u>ex vivo</u> experiments to the <u>in vivo</u> healing study.

Five groups of New Zealand white rabbits (2 – 2.5 kg) were formed with 6 eyes/group. Both eyes of each rabbit were used for this non-survival surgery.

Control 1: no RB; no light; amnion over incision for 7 min to mimic the treatment time for 100 J/cm^2
Control 2: no RB; amnion over incision; 150 J/cm^2
Experimental group 50: RB, amnion over incision; 50 J/cm^2
Experimental group 100: RB, amnion over incision; 100 J/cm^2
Experimental group 150: RB, amnion over incision; 150 J/cm^2

After anesthesia, a single 3.5-mm incision was made in the central cornea with a K blade (Katena Products, Inc). The treatment parameters for the experimental groups were those established above in the ex vivo studies: 0.1% RB, 5 min stain, irradiance = 0.25 W/cm^2, 13 mm amnion patch, 4 mm central opaque disc. Control 1 assessed sealing of the incision without light or RB. Control 2 assessed the ability of the highest fluence and amnion without RB staining to seal the incision. The IOP_L was measured immediately after the light treatment or after 7 min for the unirradiated control (Control 1).

The results are shown in Figure 6. The IOP_L for Control 2 was not greater than that for Control 1 indicating that even the highest fluence (150 J/cm^2) did not seal amnion to the cornea in the absence of RB. The IOP_L increased with increasing fluence and differed significantly from the IOP_L for Control 2 when the cornea/RB-stained amnion was irradiated with 100 and 150 J/cm^2. The IOP_L produced by 100 J/cm^2 in this in vivo study (347 ± 86 mm Hg) was greater than that produced by the same fluence using ex vivo eyes (144 ± 49 mm Hg; Figure 4). However, when the control IOP_L values were subtracted from the experimental values the difference was not statistically significant (experimental – control 2 in vivo = 205 ± 86 mm Hg; experimental – control 2 ex vivo = 134 ± 45 mm Hg). The higher IOP_L for controls in vivo may be due to the greater stiffness of the cornea on in vivo eyes, compared to frozen ex vivo eyes, that holds the incision closed.

There was no significant difference between the IOP_L values after treatment with 100 and 150 J/cm^2. This result contrasts with that obtained for ex vivo photobonding (Figure 4), which showed a greater IOP_L at the higher fluence. Possibly this contrast is due to the greater variability in the IOP_L in vivo at 150 J/cm^2. This study was designed to include 6 eyes/group. However, technical difficulties with the IOP measurement required that results for 2 eyes from Control 2, and 1 eye each from 50 and 100 J/cm^2 groups be eliminated from the analysis.
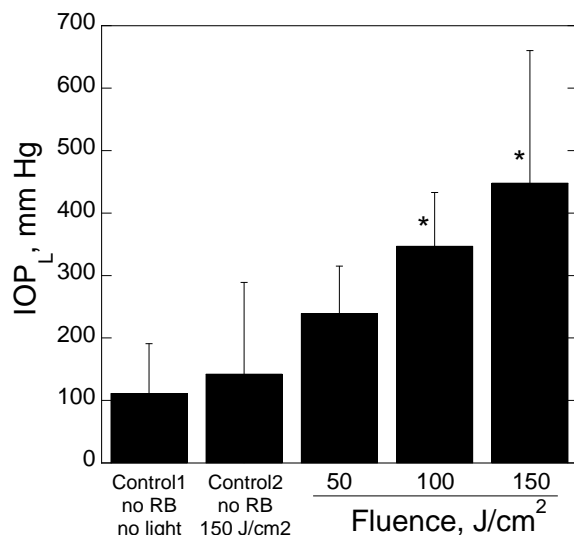

Figure 6. In vivo study of photoactivated bonding of amnion patch to de-epithelialized rabbit corneas. In the experimental groups, amnion was stained with 0.1% RB, placed over the incision and treated with an irradiance of 0.25 W/cm^2 for varying times to deliver the fluences shown. n = 5/group
* p < 0.5 compared to Control 2.

These results indicate that RB-stained amnion is strongly bonded to rabbit cornea in vivo. An IOP of 347 ± 86 mm Hg was required to break the seal after treatment with 100 J/cm^2 (6.7 min at 0.25 W/cm^2.) Increasing the fluence to 150 J/cm^2 did not significantly increase the bonding strength.

1.c. Compare healing after PTB and suture treatments

The animal study protocol was approved on April 16, 2010 by the USAMRMC ACURO for the use of rabbits and was approved by the Brooke Army Medical Center IACUC ((IACUC Protocol Number A-2009-02). The healing studies after PTB bonding to rabbit cornea have been delayed because of the late opening of the new USA ISR vivarium. These facilities are now scheduled to open in late September, 2010. The laser and other equipment have been ordered, technical staff is being hired and arrangements are being made to obtain a commercial supply of amniotic membrane. A more complete description of the activities at Brooke Army Medical Center is included in the Annual Report for the partnering Grant Agreement by Col Anthony J. Johnson, MD, PI.

Task 5. Design, construct and test safe light delivery systems for direct bonding of corneal injuries.

5.a. Design light delivery system

In the studies described above (Task 1) an opaque disc is placed over the pupil to block 532 nm light from reaching the retina through the pupil. However, in studies to be performed in Year 2 (Task 2), 532 nm light will be used to directly treat injuries in the central cornea after applying RB. Thus, some light will enter through the pupil and reach the retina. Light at 532 nm can cause thermal damage to retinal photoreceptor cells at sufficiently high irradiances and radiant exposures, and can also lead to photochemical damage directly to the retinal photoreceptors. Light exposure standards for exposure of retina to cw lasers have been compiled in the American National Standard for Safe Use of Lasers (ANSI 136.1) (10).

A program developed by Drs. Webb (co-Investigator) and Delori (Consultant) was used to calculate the irradiance at the retina from knowledge of the laser power, irradiation time and wavelength and to compare the result with the appropriate ANSI standard (11). ZEMAX software for optical system design was used to design optics that delivers an amount of light that is safe for the retina when using the laser parameters that are expected to seal a corneal incision. The irradiation parameters selected were based on the results of our previous study of photoactivated bonding of cornea surgical incisions (4). The input parameters for calculating the retinal radiant exposure (called fluence by photobiologists) and the retinal irradiance were: 532 nm, retinal image diameter = 7 mm, corneal irradiance = 0.25 W/cm^2, pupil diameter = 2 mm, exposure duration = 400 sec. The calculated retinal radiant exposure was 8.16 J/cm^2, well below the ANSI standard of 33.4 J/cm^2. The calculated retinal irradiance was 20.4 mW/cm^2, which is well below the ANSI standard of 83.6 mW/cm^2.

As shown in Figure 7, the light emerging from the fiber is collimated using a 20X microscope objective (na 0.4). That forms a 12 mm diameter image of the fiber end, but since the light at this image is collimated, it cannot be used to irradiate the cornea. If it were, that light would be focused at the retina, causing damage. Rather, a 30° diffuser at the image plane changes the collimated beam to a diverging one of the same diameter. A pair of Freznel lenses (21.6 and 50.8 mm focal length) focuses the image of the diffuser on the cornea. The requisite irradiance

thus falls on the cornea, but light passing beyond the cornea is strongly divergent so that it spreads safely over a wide area of the retina.
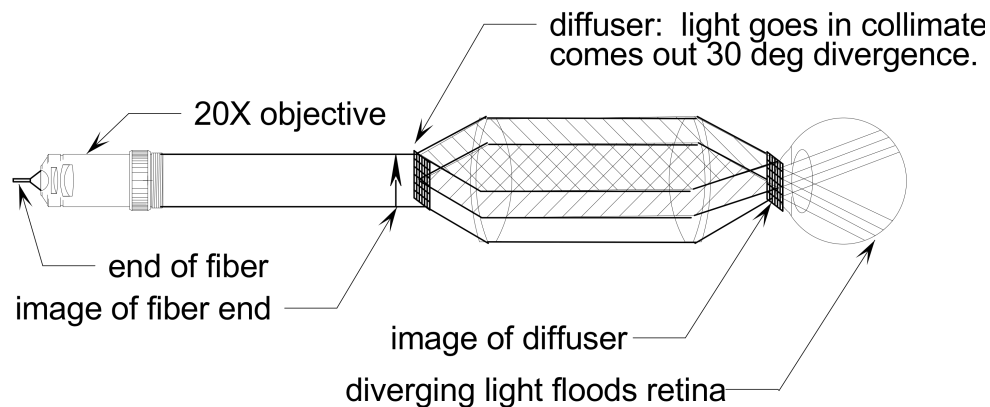

Figure 7. Schematic diagram of light delivery system.

To confirm that this design achieves our goal, we measured the laser power at the corneal plane, using a NOVA power meter (Ophir Optronics Ltd., North Andover MA). The input power from the fiber optic was 0.36 W. At the image of the diffuser on the cornea (1.3 cm diameter) the power was 0.22 W, ~40% loss of power through the optics. The irradiance on the cornea was 0.17 W/cm^2 (lower than the 0.25 W/cm^2 used in our previous studies) and, consequently, safe by ANSI standards. In the next year, we will increase the input laser power and use this optical system to seal corneal incision with PTB.

We have designed a light delivery system that is safe for sealing of cornea wounds using RB and 532 nm light, constructed the first model of this system and validated that the power delivered does not exceed the ANSI standard for retinal damage.

KEY RESEARCH ACCOMPLISHMENTS

• Identified the treatment parameters that produce strong bonding of a Rose Bengal-stained amnion patch over corneal wounds in ex vivo rabbit eyes. The amnion patch size, dye concentration, dye staining time and the irradiance and fluence of green light were systematically varied to chose the parameters for the in vivo photobonding study.

• Determined that a fluence of 100 J/cm^2 of 532 nm (green) laser light strongly bonds a Rose Bengal-stained amnion patch over a penetrating corneal injury in the central cornea in vivo. The seal formed resists opening at intraocular pressures up to 350 mm Hg.

• Designed and constructed the first version of a light delivery system for direct light-activated bonding of penetrating injuries in the central cornea when an amnion patch is not used. Measurements indicate that the irradiance is below the thresholds for retinal damage at the fluences that produce tissue bonding.

REPORTABLE OUTCOMES
1. Rose Bengal photosensitized crosslinking of collagen for repair of penetrating eye injuries E. Verter, M. Yao, A. Blanden, A.J. Johnson, R.W. Redmond and I.E. Kochevar, Invited Presentation at the American Society for Photobiology Annual Meeting, June 12-16, Providence RI

2. Light-activated technology for repair and regeneration after traumatic injury. I. E. Kochevar and R.W. Redmond, ATACCC 2010 Conference, August 16-19, St. Pete Beach FL (Poster # RM14).

CONCLUSIONS
In this first reporting period, we completed the initial phase of developing a simple and rapid light-initiated tissue bonding technology to decrease vision loss and ocular complications after penetrating eye injuries. Rapid closure of these wounds is critical to preventing infection and stabilizing the eye for further surgery. Current methods have important drawbacks: suturing is tedious, time-consuming and can damage the tissue; fibrin glues are not strong enough; and cyanoacrylate glues can cause damage upon removal and interfere with subsequent surgery.

In Year 1 we established the feasibility of sealing amniotic membrane over penetrating corneal lacerations in vivo (rabbit eyes) using a sutureless, glueless technology. The seal withstood an intraocular pressure of 350 mm Hg, more than 10 times higher than the normal intraocular pressure. The treatment parameters are clinically relevant (6.5 min light treatment), suggesting that translation to the clinic is feasible. Studies designed to increase the efficiency of the light-activated process may be able to decrease the treatment time. We also designed and built a model light delivery system that, by calculations, will allow treatment without retinal damage.

Thus, the results obtained from these studies did not identify problems or obstacles that will inhibit the translation of this light-activated repair technique to clinical use. In addition, this treatment involves off-label use of three FDA-allowed devices (clinical KTP laser, Rose Bengal dye, human amniotic membrane), which may facilitate receiving FDA clearance. Safety studies, particularly of the laser, will be required but are not included in this project. This should be addressed.

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APPENDICIES

Meeting abstracts:

1. Rose Bengal photosensitized crosslinking of collagen for repair of penetrating eye injuries E. Verter, M. Yao, A. Blanden, A.J. Johnson, R.W. Redmond and I.E. Kochevar, Invited Presentation at the American Society for Photobiology Annual Meeting, June 12-16, Providence RI

2. Light-activated technology for repair and regeneration after traumatic injury. I. E. Kochevar and R.W. Redmond, ATACCC 2010 Conference, August 16-19, St. Pete Beach FL (Poster # RM14).

Rose Bengal photosensitized crosslinking of collagen for repair of penetrating eye injuries

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We are developing a rapid, simple method for tissue repair that may replace sutures in many types of surgery. Rose Bengal (RB) photosensitization is used to initiate formation of covalent crosslinks between extracellular matrix proteins between tissue surfaces in a process called Photochemical Tissue Bonding (PTB). Although RB is widely known to be phototoxic to cells in vitro, our previous study indicated that PTB is not cytotoxic when used to bond skin in vivo.

In this study, we used PTB to seal penetrating eye injuries. V-shaped incisions were made centrally in the cornea of ex vivo rabbit eyes. A circular "patch" of human amniotic membrane (13 mm diameter) was stained on the stromal side with RB and placed over the corneal incision. A small opaque disc (4 mm diameter) was placed over the central cornea. The corneal surface was exposed to green light from a 532 nm KTP laser to bond the amnion to the cornea surface. The strength of the seal was measured by infusing saline into the anterior chamber and determining the intraocular pressure that produced leakage (IOP_L) at the wound site. The IOP_L increased with increasing RB concentration (0.01 – 1%) and with increasing light fluence (25 – 150 J/cm^2). Treatment with 0.1% RB in PBS and 100 J/cm^2 produced an IOP_L of 250 mm Hg, which is >10-fold higher than the IOP in normal eyes. Purging the cornea + amnion with nitrogen during the irradiation in a special chamber inhibited bonding. Chemically blocking amnion protein lysines, which are frequently involved in protein crosslinks, did not alter the PTB-induced bond strength. These results indicate that PTB may be a useful clinical approach for sealing penetrating eye injuries.

Light-activated technology for repair and regeneration after traumatic injury

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We have developed a light-activated tissue repair technology to attach tissue surfaces and create engineered tissues that is applicable to repair of traumatic injuries. The technology, called Photo Tissue Bonding (PTB), is based on photochemical crosslinking of tissue proteins and produces an immediate, water-tight and strong bond. PTB has been successfully used in preclinical studies to seal corneal incisions and transplants, seal skin incisions and to reattach peripheral nerves, small blood vessels and tendons. A pilot clinical study to skin excisional

wounds demonstrated that PTB produced less scarring than epidermal sutures. Light-activated crosslinking of proteins has also been used in the generation of neocartilage.

In the PTB process, a light-sensitive dye (Rose Bengal) is applied to the tissue surfaces, the surfaces are placed in contact and the dye-stained area is exposed to green light (532 nm; KTP laser) for a few minutes without causing thermal damage. Recent results from three studies will be presented.

   1) Peripheral nerve repair: Neurorraphy of severed rabbit peroneal nerve using PTB to seal a wrap of RB-coated human amnion over the repair site demonstrated greater histological and electrophysiologic recovery than sutured repair. This result suggests that PTB seals and isolates the internal nerve environment for optimal nerve regrowth as well as reducing needle trauma. 2) Sealing skin excisional wounds: In a pilot clinical study, PTB was compared with sutures for superficial closure of excisional wounds (collaboration with S. Tsao, MD). In 31 wounds, after placement of deep sutures, one half was closed with sutures and one half closed with PTB. At 2 weeks post surgery the PTB-closed side showed much less inflammation than sutured closure and at 6 months showed less scarring. 3) Sealing penetrating eye injuries: PTB was used to seal amnion over corneal and scleral wounds in rabbit eyes ex vivo (collaboration with Col A.J. Johnson MD). Wounds sealed with PTB resisted intraocular pressures up to 200 mm Hg.